Antioxidant and Hepatoprotective Activity of Fruit Extracts of *Tetrapleura tetraptera* (Schum & Thonn) Taubert

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Abstract

The antioxidant and hepatoprotective activity of *Tetrapleura tetraptera* extracts in carbon tetrachloride (CCl_4)-induced liver injury in rats was investigated. *T. tetraptera* extracts showed varying levels of protective action against CCl_4-induced liver damage as evidenced through the significant reduction in the activities of serum marker enzymes for liver damage (alanine transaminase, aspartate transaminase, and alkaline phosphatase), and bilirubin levels when compared with CCl_4-intoxicated control rats. The extracts decreased the elevation in the activities of the enzymes in the liver. They also protected against CCl_4 induced lipid peroxidation. The extracts reduced CCl_4-liver induced necrosis in dose dependent manner. These results indicated that fruit extracts of *T. tetraptera* possess a hepatoprotective property against CCl_4-induced liver damage which was mediated through its antioxidative defenses.

Keywords: *Tetrapleura tetraptera*, Antioxidant, Hepatoprotective, Serum marker enzymes, Vitamins.

1. Introduction

Oxidative Stress is a term used to describe the oxidative damage of cells, tissues, or organs, caused by the Reactive Oxygen Species (ROS) (Noda and Wakasugi, 2001). It is caused by an imbalance between the production of reactive oxygen species and the detoxifiers (antioxidants). The most common source of ROS is the leakage of activated oxygen from mitochondria during normal oxidative respiration. Enzymes capable of producing superoxide are xanthine oxidase, reduced nicotinamide adenine dinucleotide phosphate oxidases and cytochrome P450. ROS play an important role in cell signaling and are constantly cleared by the auto antioxidants such superoxide dismutase, catalase, glutathione peroxidase and glutathione-s-transferases. Oxidative stress has been linked to disease conditions like inflammation (Cai *et al.*, 2004), cancer (Banerjee *et al*., 2005), diabetes, cataracts and aging (Halliwell and Gutteridge, 1999). The liver is one of the largest organs in human body and the chief site for intense metabolism and excretion. Liver helps in maintaining and regulating the homeostasis of the body.

Tetrapleura tetraptera (Schum & Thonn), Taubert (*Fabeceae*) is a tree that grows in the tropical deciduous forest of West Africa, extending from Senegal to West Cameroon; it is also found in Sudan, Uganda and Zaire (Burkill, 1995). The aqueous or alcoholic decoctions of the plant are used in herbal medicine for the management of convulsion, leprosy, inflammation and or rheumatoid pains (Dalziel, 1948). Extracts of *T. tetraptera* have been reported to have antischistosomiasis (Adetunji, 2007), antiulcer (Kota *et al*., 2012), antimicrobial (Ekwenye and Okorie, 2010), contraceptive (El-Izzi *et al*., 1990) and anticonvulsant (Akah and Nwambie, 1993) activities. The fruits and seeds of *T. tetraptera* are used in the Niger Delta region of Nigeria for various ailments and have a significant antiplasmodial effect (Lekana-Douki *et al*., 2011; Igwe *et al*., 2012). The anti-inflammatory and hypoglycaemic effects of *T. tetraptera* (Taub) fruit aqueous extract in rats has been investigated using egg albumin-induced pedal oedema and streptozotocin (STZ)-induced diabetes mellitus (Ojewole and Adewunmi, 2004). The pods notably have an appealing culinary use for mothers from the first day of delivery to post parturition and as a lactation aid (Ekwenye and Okorie, 2010). Here we investigated the antioxidant and hepatoprotective activities of the fruit extracts of *T. tetraptera*.

2. Materials and methods

2.1. Material

The fruits of *Tetrapleura tetraptera* were purchased in Ogige Market Nsukka, Enugu State. The chemicals used for this work were purchased from Sigma-Aldrich, Germany. Serum alanine aminotransferase (ALT) and Serum aspartate aminotransferase (AST) kits

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(Randox,U.K), alkaline phosphate (ALP) kit (Quimica Clinica Aplicada, Spain), serum bilirubin kit (Boehringer Mannheim GmbH, Diagnostica, Germany) and UV/Visible Spectrophotometer (Perkin Elmer, Lambda 25) were used in the study.

2.2. Extraction Procedure

Five hundred grams (500 g) of the pulverized fruit of *T. tetraptera* were extracted with *n*–hexane (1.0 L). The marc was dried and re-extracted with methanol (1.0 L) using Soxhlet method. The extracts were concentrated *in vacuo* at 40°C and freeze-dried to produce crispy extracts.

2.3. Animals

Wistar rats and mice of both sexes were obtained from the Department of Veterinary Medicine, University of Nigeria Nsukka. The animals were maintained on standard pellet diet and tap water *ad libitum* and acclimatized for 14 days before use. The use and care of laboratory animals were conducted in accordance with the best internationally accepted practices that are contained in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) and approved by the local Ethics Committee of our institution.

2.4. Design for Hepatoprotective Assay

The rats were divided randomly into nine groups of six rats each. The hepatoprotective activity of the plant extracts was tested using CCl_4 model. Group I (normal control) received neither the plant extract nor CCl_4 for 5 days, i.e., they received only food and water. The rats were treated with the extracts as a fine suspension in 3% Tween 80 for 5 days. The test samples were administered to rats for 2 days before the injection of CCl_4 on the third and fourth days.

Group I: Served as normal control and received only food and water.

Group II: Served as induction control and received 0.75 mg/kg b.wt of CCl_4

Group IIIA: Received 100 mg/kg b.wt of *n*-hexane extract

Group IIIB: Received 300 mg/kg b.wt of *n*-hexane extract

Group IIIC: Received 500 mg/kg b.wt of *n*-hexane extract

Group IVA: Received 100 mg/kg b.wt of methanol extract

Group IVB: Received 300 mg/kg b.wt of methanol extract

Group IVC: Received 500 mg/kg b.wt of methanol extract

Group V: Served as positive control and received Vitamin E (1000 UI)

2.5. Assay for Hepatoprotective activity of the Extracts

The hepatoprotective activity of the extracts were assessed biochemically and histopathologically

2.5.1. Biochemical evaluation of ALT and AST

The animals were treated for 5 days and sacrificed under chloroform anesthesia. Blood samples from each rat were withdrawn directly by heart puncture with syringes into properly labeled EDTA bottles and allowed to clot for 1 h at room temperature. Serum was separated by centrifugation at 3000 rpm and 40°C for 20 min. The separated serum was used for the estimation of the biochemical parameters like Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and bilirubin. Serum ALT and AST were determined colorimetrically according to the method of Reitman and Frankel (1957) with slight modification. Briefly, ALT and AST determination was done by monitoring the concentrations of pyruvate hydrazone formed with 2, 4 dinitrophenyl hydrazine. 0.5 ml of Glutamic-Pyruvic Transaminase (GPT) substrate phosphate buffer solution was added into test tubes labeled blank, sample, control blank and control, respectively, for ALT and AST, respectively. 0.1 ml serum sample was added to the test tubes and the tubes were incubated at 37°C for 30 min. A volume of 0.5 ml each of 2, 4-Dinitrophenylhydrazine was added into all the test tubes. 0.1ml of sample and control was added into their respective blank test tube. The contents of each test tube was mixed and allowed to stand for 20 min at 20-25°C. 5.0 ml of 0.4N sodium hydroxide was added to each tube, mixed and their absorbencies read at 546 nm against the respective blank prepared.

2.5.2. Biochemical evaluation of Alkaline Phosphatase

Alkaline Phosphatase activity was done by Phenolphthalein monophosphate method (Braide *et al.*, 2011). Briefly, test tubes were respectively labeled sample, standard and control. A volume of 0.1 ml of distilled water was pipette into the test tubes followed by a drop of the substrate (Phenolphthalein monophosphate) into each of the test tubes. The contents in the tubes were mixed and incubated at 37°C for 5min in a water bath. A volume of 0.1 ml of sample, standard and control were added into the respective test tubes and the tubes incubated at 37°C for 20 min. A volume of 5.0 ml of color developer was added to each test tubes, mixed, and their absorbance read at 550 nm using water as blank. The activity of sample was calculated using the absorbance of sample against absorbance of standard multiplied by concentration of standard (Babson *et al.*, 1966).

2.5.3. Biochemical evaluation of Bilirubin

The total and direct bilirubin activity was assessed by colorimetric method as outlined in the diagnostic kit described by Sunday *et al.* (2014).

2.5.4. Histopathological study

For the histopathological study, liver from each rat was removed, sliced and preserved in 10% neutral formalin solution for 7 days. Thin sections of the liver were washed and dehydrated in ethanol and subsequently immersed in xylene solution for 15 mins. Then representative blocks of the liver tissues were taken and possessed for paraffin embedding using the standard microtechnique (Ahsan *et al.*, 2009). Thin sections (5 µm) of the livers were stained with haematoxylin and eosin (H & E) and observed microscopically for histopathological studies.

2.6. Assay for Antioxidant activity

The lipid peroxidation was determined as ThioBarbituric Acid-Reactive Substance (TBARS) as described by Atawodi et al. (2014) based on the principle that peroxide intermediates generated release malondialdehyde (MDA) upon cleavage (Torres et al., 2004). The thiobarbituric acid-reactive substance and malondialdehyde react with thiobarbituric acid to give a red or pink adduct which absorbs maximally at 532 nm. One ml of serum was added 0.45 ml of normal saline and mixed thoroughly before adding 0.5 ml of 25% trichloroacetic acid and 0.5 ml of 1% thiobarbituric acid. A blank was prepared with distilled water. The mixture was incubated at $95^{o}C$ for 40 min in a water bath followed by centrifugation at 3000 rpm for 10 min. MDA was measured colorimetrically at 532 nm and the level of thiobarbituric acid reactive substances were quantified as lipid peroxidation products by referring to a standard curve of malondialdehyde (MDA) concentration (Asuku et al., 2012).

2.7. Statistical Analyses

A statistical analysis of the results was performed using one-way analysis of variance (ANOVA), using GenStat 7.22 Discovery Edition 3 (VSN International Limited). All values were expressed as mean $\pm$ S.D., and a value of $P < 0.05$ was considered significant as compared to the respective control group.

3. Results

3.1. Biochemical and antioxidant parameters

Three doses of the n-hexane and methanol extracts of Tetrapleura tetraptera were evaluated for their antioxidative and hepatoprotective activities in carbon tetrachloride (CCl_4)-induced liver injury in rats. It was observed that the extracts reduced the level of liver function biomarker (ALT, AST, ALP, and bilirubin) and antioxidant parameter (MDA) compared with the CCl_4 group. The levels of the biochemical parameters (ALP, AST, ALT) in the sera were elevated in CCl_4 treated animals, indicating liver damage. Also, the levels of these biochemical parameters in the rats treated with Tetrapleura tetraptera fruit extracts showed a remarkable reduction (Table 1 and 2). The LPO levels by thiobarbituric acid reaction showed an increase in LPO in the CCl_4 treated rats. Treatment with Tetrapleura tetraptera fruit extracts at 100-500 mg/kg significantly ($P < 0.005$) reduced the LPO level comparable to the normal control (Table 3).

Table 1. Effect of extracts of Tetrapleura tetraptera, on various biochemical parameters in rats with carbon tetrachloride induced hepatotoxicity.

Treatment	Doses (mg/kg)	ALP (IU/L)	AST (IU/L)	SGPT (IU/L)
Normal control	-	50.67$\pm$14.36	10.33$\pm$2.98	8.6$\pm$1.75
CCl$_4$	0.75	107.0$\pm$7.73	20.0$\pm$3.53	17.59$\pm$5.24
CCl$_4$ + n-hexane extract	100	54.50$\pm$7.79*	15.50$\pm$3.64	16.67$\pm$2.63*
	300	54.03$\pm$6.39	10.90$\pm$2.23	14.0$\pm$3.81
	500	50.50$\pm$16.46	7.10$\pm$0.64	12.20$\pm$1.73
CCl$_4$ + Methanol extract	100	66.00$\pm$19.05*	13.40$\pm$1.30	16.96$\pm$5.01*
	300	57.53$\pm$14.06	11.40$\pm$4.16	14.23$\pm$5.11
	500	56.23$\pm$9.73	10.50$\pm$4.85	11.85$\pm$5.08
Vitamin E	1000 IU	66.47$\pm$6.67*	11.17$\pm$3.22	12.93$\pm$3.74

All values are mean $\pm$SD, n=6 rats in each group. *P<0.005 as compared with the normal control.

Table 2. Total and direct bilirubin levels in rats treated with extracts of T. tetraptera fruits for 5 d.

Treatment	Dose (mg/kg)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
Normal control	-	1.03$\pm$0.26	0.93$\pm$0.66
CCl$_4$	0.75	1.85$\pm$0.26	0.37$\pm$0.05
CCl$_4$ + n-hexane extract	100	1.34$\pm$1.65	0.27$\pm$0.33*
	300	1.10$\pm$0.00	0.22$\pm$0.00
	500	0.93$\pm$1.58	0.19$\pm$0.31*
CCl$_4$ + Methanol -extract	100	1.35$\pm$0.43	0.27$\pm$0.09
	300	1.18$\pm$0.57	0.24$\pm$0.12*
	500	1.13$\pm$0.74	0.23$\pm$0.07*
Vitamin E	1000 IU	1.05$\pm$0.31	0.21$\pm$0.07

All values are mean $\pm$SD, n= 6 rats in each group. *P<0.005 as compared with the normal control.

Table 3 Malondialdehyde (MDA) levels in rats treated with extracts of T. tetraptera fruits for 5 d.

Treatment	Dose (mg/kg)	TBARS (µg/ml)
Normal control	-	6.25$\pm$0.00
CCl$_4$	0.75	14.17$\pm$11.80
CCl$_4$ + n-hexane extract	100	9.50$\pm$2.96*
	300	7.00$\pm$4.76
CCl$_4$ + methanol extract	100	12.36$\pm$2.82
	300	9.75$\pm$2.49*
	500	8.86$\pm$4.12
Vitamin E	1000 IU	9.67$\pm$1.84

All values are mean $\pm$SD, n= 6 rats in each group. *P<0.005 as compared with the normal control.

3.2. Histological Observations

The histopathological study of the liver treated with CCl_4 and the extracts are shown in Figure 1. The CCl_4 treated rat liver shows a damage of hepatocytes with hepatocellular vacuolization, focal necrosis and congestion of hepatic sinusoids while the liver from the extract treated rats showed a mild vacuolization.
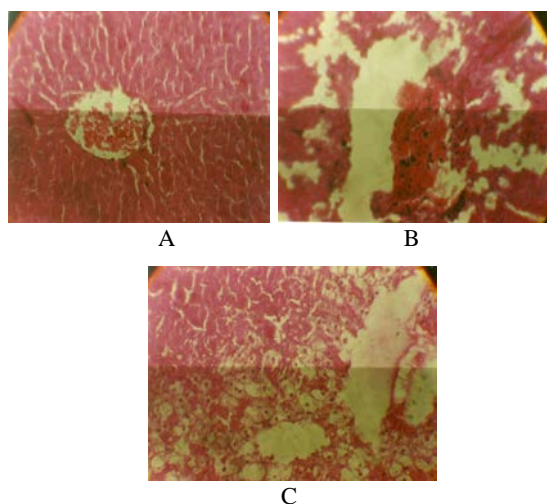

Figure 1. Histopathology of (a): normal liver having histological structures of normal hepatic lobules; (b): Toxicant treated liver (CCl_4, 0.75 mg/kg body weight) showing damage to hepatocytes with hepatocellular vacuolization, focal necrosis and congestion of hepatic sinusoids;(c): *Tetrapleura tetraptera* (methanol extract) treated liver (200 mg/kg body weight) showing mild vacuolization. H & E x400.

4. Discussion

The liver is very susceptible to damage by xenobiotics and non-xenobiotics induced oxidative stress. Liver damage is manifested in increased serum levels of aspartate transaminase (AST) or serum glutamate oxaloacetate transaminase (GOT), alanine transaminase (ALT) or serum glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP) and serum bilirubin, as well as pronounced necrosis of hepatic cells. Carbon tetrachloride (CCl_4) induced hepatotoxicity model is widely used for the study of hepatoprotective effects of drugs and plant extracts (Ahsan *et al*., 2009). Treatment of animals with *T. tetraptera* extracts and Vitamin E significantly reduced the level of liver function biomarker (ALT, AST, ALP, and bilirubin) and antioxidant parameter (MDA) compared with the CCl_4 group. The test extracts mediated reduction in levels of AST, ALT as well as repair of hepatic tissue damage caused by CCl_4. These effects are in agreement with the commonly accepted view that serum levels of transaminase return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew *et al*., 1987). Liver damage is manifested in increased serum levels of ALP, AST, ALT and bilirubin; as well as, pronounced necrosis of hepatic cells. The rats that received the extracts had a significant decrease in serum ALP, AST, ALT and bilirubin when compared with those of the normal control

rats (Tables 1-2). These indicate that the extracts have a hepatoprotective effect that was found to be more pronounced at higher doses. The *n*-hexane extract showed a higher hepatoprotective activity than the methanol extract. Studies have shown that phenolic compounds and flavonoids present in plant extracts enhance their hepatoprotective activities (Maheswari *et al*., 2008). Thus, the observed hepatoprotective activity of the fruit extract could be attributed to the presence of flavonoids in the fruit extract.

Lipid peroxidation induced by CCl_4 is a commonly used experimental animal model for studying oxidative injury in biological systems (Srilakshmi *et al*., 2010). Cytochrome P-450 enzymes are believed to metabolize CCl_4 to trichloromethyl radicals that can initiate peroxidation of unsaturated fatty acid and initiate chain reactions of lipid peroxidation. The lipid solubility of CCl_4 allows it to cross the cell membrane and to be distributed to the organs. However, the liver is the major target organ of CCl_4–induced toxicity owing to its high content of cytochrome P-450 enzyme. Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates a number of degradation products. During lipid peroxidation, malonaldehyde (MDA), one of the thiobarbituric acid reactive substances (TBARS) is produced and has been studied widely as an index of lipid peroxidation and as a marker of oxidative stress (Njayou *et al*., 2013). In the detoxification process of CCl_4 from the body, free radicals were generated which can initiate lipid peroxidation and reactions leading to the generation of thiobarbituric acid reactive substance (TBARS). The extracts lowered TBARS in the sera of treated rats compared with those of the normal control rats. The *n*-hexane extract lowered TBARS more than the methanol extract (Table 3). This is an indication that the extracts have antioxidative potential. The fruit extracts of *T. tetraptera* lowered the formation of TBARS in a concentration-dependent manner. This suggests that the fruit extracts prevent the oxidation of cell membranes lipids. Secondary metabolites like flavonoids have been isolated from the stem bark of *Tetrapleura tetraptera* (El-Izzi *et al*., 1990). Flavonoids have been reported to have antioxidant activity (Kostić *et al*., 2013). The antioxidative activity reported in our study could be attributed to these flavonoids present in the fruit.

The result of the histopathological studies provided supportive evidence for biochemical analysis. Histology of liver section of normal control rats exhibited normal hepatic cells each with well defined cytoplasm, prominent nucleus and nucleolus and well brought out central vein whereas that of CCl_4 intoxicated group animal showed total loss of hepatic architecture with hepatocellular necrosis and wide area of hyperaemia. The liver section of the rats treated with 300 mg/kg b.wt methanol extract showed patches of hepatic cells undergoing mild degeneration and produced mild necrosis of hepatocytes in the portal areas (Figure 1).

5. Conclusion

The *n*-hexane and methanol extracts of *Tetrapleura tetraptera* have both antioxidative and hepatoprotective properties, which have been attributed to presence of secondary metabolites in the extracts. Hence further studies are required for the standardization of these fruit extracts.

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